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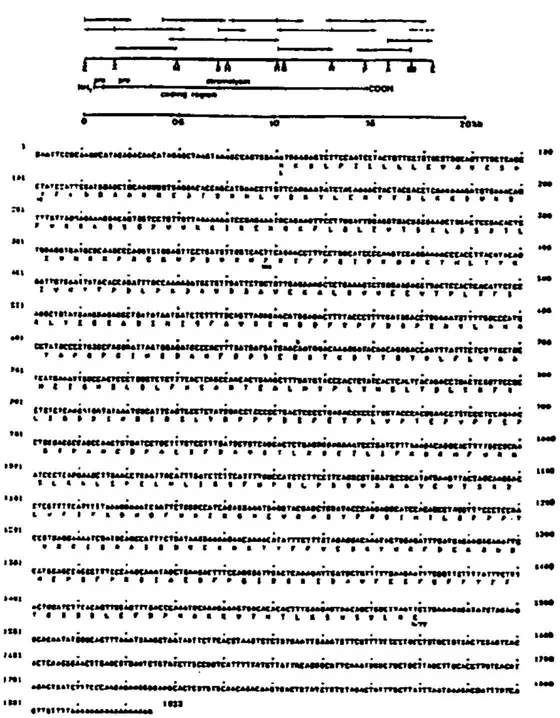
(57) Abstract

Process for the production of a mammalian stromelysin by a recombinant DNA process. The invention also relates to specific proteins, DNA sequences, vectors, host organisms, pharmaceutical compositions, DNA probes, antibodies and to the use of stromelysin to enhance the activity of collagenase. Stromelysin may be useful therapeutically for example in the debridement of dermal ulcers, modification of scar tissue formation arising from healing of wounds and in the treatment of herniated vertebral discs.



PROCESS FOR THE PRODUCTION OF A PROTEIN

Field of the Invention

This invention relates to a process for the production of a protein, particularly a metalloproteinase by a recombinant DNA process. The invention also relates to specific proteins, DNA sequences, vectors, host organisms and to pharmaceutical compositions.

Background to the Invention

The metalloproteinases are a family of enzymes produced in mammalian tissues and which are believed to play an important role in the resorption of the extracellular matrices of connective tissues.

The family of metalloproteinases includes collagenase, gelatinase and stromelysin which work together synergistically to digest all the major macromolecules comprising extracellular matrices. The enzymes are found in latent proenzyme form activatable by trypsin or 4-aminophenylmercuric acetate (APMA). The metalloproteinases have a zinc atom at their active site and require calcium for full activity.

Usually tissue metalloproteinases are coordinately synthesised, and for many tissues a specific stimulus is needed to induce synthesis (Reynolds J. Brit. J. Dermatology (1985) 112 715-723). The metalloproteinases are present naturally in low concentration and cannot be extracted in large amounts from mammalian tissues.

Of particular interest is stromelysin which is known to degrade many components of the connective tissues such as proteoglycan core protein, non-helical regions of type IV collagen, laminin, fibronectin, elastin, gelatin and procollagens types I, II and III and which may play a role in biological processes such as wound

healing. Stromelysin may be useful therapeutically in processes such as debridement of dermal ulcers and modifying scar tissue formation arising from the healing of wounds such as burns or necrosis. Stromelysin may also be used in the treatment of herniated vertebral discs to effect dissolution of nucleus pulposus. In order to use stromelysin in this way it must be produced on a large scale and at a commercially viable cost. We provide mammalian stromelysin in such commercially worthwhile amounts by producing it in accordance with the invention, using recombinant DNA techniques.

Preprostromelysin is a large molecular weight form of stromelysin which on secretion is processed to yield the lower molecular weight prostromelysin. Activation of prostromelysin by, for example, treatment with 4-amino phenylmercuric acetate yields the mature biologically active stromelysin.

Summary of the Invention

According to the present invention there is provided a process for the production of a mammalian stromelysin comprising culturing host cells transformed with a DNA sequence coding for the stromelysin.

The term mammalian stromelysin as used herein denotes a mammalian stromelysin having the amino acid sequence of an authentic mammalian stromelysin, an analogue thereof, or a biologically active peptide fragment of either of these, having the biological activity associated with authentic mammalian stromelysin.

In a preferred embodiment the invention provides a process for the production of human stromelysin comprising culturing host cells transformed with a DNA sequence coding for human stromelysin.

The process allows the production of relatively large quantities of stromelysin and, for the first time, facilitates complete characterisation of the structure and pharmacological properties of the protein.

The stromelysin preferably has an amino acid sequence of greater than 90 percent homology (common amino acids/total amino acids) with the sequence of amino acids 1 to 477 of human preprostromelysin as shown in Figure 4 of the accompanying drawings. More preferably, the homology is greater than 98 per cent and most preferably the human preprostromelysin has the amino acid sequence substantially as shown in Figure 4.

In addition to the complete mammalian stromelysin proteins, it is envisaged that relatively small peptide fragments of such proteins e.g. a peptide fragment of stromelysin may be useful as a metalloproteinase. Thus, in a particular embodiment of the first aspect of the invention, and where appropriate in subsequent aspects of the invention, the term stromelysin includes a peptide fragment of a stromelysin protein.

The mammalian stromelysin is suitably a methionine-stromelysin or a methionine-prostromelysin. It is currently understood that to obtain expression of a DNA sequence, the DNA sequence must possess a 5' ATG codon and the corresponding polypeptide therefore possesses an N-terminal methionine amino acid. As used herein the term "methionine-stromelysin" and "methionine-prostromelysin" denote an authentic mammalian stromelysin or prostromelysin, (or an authentic mammalian stromelysin or prostromelysin, modified or substituted to provide a functionally equivalent protein) having an N-terminal methionine residue. Preferably the methionine residue is adjacent to the N-terminal amino acid of the stromelysin or prostromelysin but may be separated therefrom by one or more amino acids provided that the protein possesses stromelysin or prostromelysin functional activity.

In a second aspect of the invention there is provided a process for the production of a mammalian stromelysin comprising producing a precursor of the stromelysin by culturing host cells transformed with a DNA sequence coding for th stromelysin precursor and cleaving the precursor to produce the stromelysin.

In a pr ferred embodiment of the second aspect of the invention

we pr vide a process for the production of human stromelysin comprising producing a precursor of human stromelysin by culturing host cells transformed with a DNA sequence coding for the stromelysin precursor and cleaving the precursor to produce human stromelysin.

The precursor protein may be a methionine-stromelysin or methionine-prostromelysin.

The precursor protein may be a mammalian stromelysin or a prostromelysin with an amino terminal signal sequence. The signal sequence may be a sequence having the effect of encouraging transport of the expression products from the host cell in which the DNA sequences have been expressed. For example, in the case of human stromelysin, a signal sequence of amino acids such as depicted by amino acids 1 to 17 in Figure 4 of the accompanying drawings may be attached to the amino terminal amino acid to form preprostromelysin. This signal sequence assists in the export of the product from eukaryotic host cells and is itself cleaved from the product during transport through the cell membrane.

The precursor protein may be a fusion protein comprising a heterologous protein and a mammalian stromelysin or prostromelysin protein. The heterologous protein may be all or a part of a protein capable of being produced in a host organism, preferably at a high level. Such heterologous proteins include β-galactosidase, chloramphenicol acetyl transferase (CAT) and the product of the <u>trpE</u> gene. The fusion protein preferably includes a site susceptible to selective chemical or enzymatic cleavage between the stromelysin or prostromelysin protein and the heterologous protein. The heterologous protein may be a yeast signal sequence and the host organism may be a yeast. In this preferred embodiment, the yeast host organism advantageously cleaves the fusion protein to produce a mature stromelysin or prostromelysin.

The process of the invention permits the production of essentially pure human stromelysin, prostromelysin or prepro-stromelysin. The term essentially pure is used to denote stromelysin which is essentially free from other proteins of human origin.

In a third aspect the invention th refore provides an essentially pure mammalian stromelysin, pr stromelysin or preprostromelysin characterised in that the mammalian stromelysin, prostromelysin or preprostromelysin is human stromelysin, prostromelysin or preprostromelysin.

In a preferred embodiment of the third aspect of the invention the essentially pure human stromelysin has substantially the amino acid sequence of from 100 to 477 as shown in Figure 4 of the accompanying drawings, the essentially pure human prostromelysin has the amino acid sequence of from 18 to 477 as shown in Figure 4 of the accompanying drawings and the essentially pure human preprostromelysin has the amino acid sequence of from 1 to 477 as shown in Figure 4 of the accompanying drawings.

In a fourth aspect of the invention, we provide a mammalian stromelysin or prostromelysin produced by the process of the first aspect of the invention or a mammalian stromelysin or prostromelysin precursor protein produced as an intermediate compound.

In a preferred embodiment of the fourth aspect of the invention the stromelysin is human stromelysin or prostromelysin or a precursor thereof.

In a fifth aspect of the invention, we provide a fusion protein comprising a heterologous protein and a mammalian stromelysin or prostromelysin.

In a preferred embodiment of the fifth aspect of the invention the fusion protein comprises a heterologous protein and human stromelysin or human prostromelysin.

In a sixth aspect of the invention, we provide a DNA sequence coding for the amino acid sequence of a mammalian stromelysin or prostromelysin , or a precursor thereof provided that when the DNA sequence codes for rat stromelysin the DNA sequence is not the pTR1 cDNA sequence described by Matrisian et al (EMBO Journal 4 14350-1440 (1985)). Preferably, we provide a DNA sequence coding

for human preprostromelysin having substantially the nucleotide sequence from 51 to 1484 inclusive as sh wn in Figure 4 of the accompanying drawings. Preferably we provide a DNA sequence coding for human prostromelysin having substantially the nucleotide sequence from 102 to 1484 inclusive as shown in Figure 4 of the accompanying drawings. Preferably we provide a DNA sequence coding for human stromelysin having substantially the nucleotide sequence from 348 to 1484 inclusive as shown in Figure 4 of the accompanying drawings.

In a seventh aspect of the invention, we provide an expression vector including a DNA sequence coding for a mammalian stromelysin, prostromelysin or precursor thereof. The vector is adapted for use in a given host cell by the provision of suitable selectable markers, promoters and other control regions as appropriate.

In a preferred embodiment of the seventh aspect of the invention the vector includes a DNA sequence coding for human stromelysin or prostromelysin or a precursor thereof.

In an eighth aspect of the invention, we provide host cells transformed with a vector according to the seventh aspect of the invention. The host cells may be any host organism which may be transformed with a vector including a DNA sequence coding for a mammalian stromelysin or a precursor thereof, such that expression of the DNA sequence occurs. Suitable host cells include yeasts (for example Saccharomyces cerevisiae) and mammalian cells in tissue culture (for example,hamster ovary or mouse mammary tumour cells). Preferably, where the host cell is a bacterium or a yeast, the vector includes a DNA sequence coding for methionine-stromelysin or methionine prostromelysin or a fusion protein including a stromelysin or prostromelysin and when the host cell is a mammalian cell in tissue culture, the vector preferably includes a DNA sequence coding for a prestromelysin or a preprostromelysin.

In a ninth aspect of the invention, we provide a pharmaceutical composition comprising a mammalian stromelysin preferably human stromelysin and a pharmaceutically acceptable

excipient. The pharmaceutical compositi n may be, for example, in the form of an inj ctable solution or in a form suitable for topical administration. The pharmaceutical composition may contain a stromelysin or a prostromelysin preferably human stromelysin or human prostromelysin which may be activated in vitro or in vivo to give biologically active stromelysin.

In a preferred embodiment of the ninth aspect of the invention we provide a pharmaceutical composition containing an effective amount of a mammalian stromelysin or prostromelysin , preferably human stromelysin or prostromelysin, for use in the debridement of dermal ulcers, modification of scar tissue formation arising from the healing of wounds such as burns and necrosis and in the treatment of herniated vertebral discs.

In a tenth aspect of the invention we provide a process for the production of a pharmaceutical composition comprising bringing a mammalian stromelysin or prostromelysin, preferably human stromelysin or prostromelysin into association with a pharmaceutically acceptable carrier.

In an eleventh aspect the invention provides a method of therapy comprising treating a patient with an effective amount of a mammalian stromelysin preferably human stromelysin, for instance for debridement of dermal ulcers, modification of scar tissue formation arising from the healing of wounds such as burns and necrosis or treatment of herniated vertebral discs.

Preferably, the stromelysin produced in accordance with the invention will have a useful pharmacological effect without significant antigenic reaction with the immune system. In particular the compounds may be used in the debridement of dermal ulcers, modification of scar tissue formation arising from the healing of wounds such as burns and necrosis and in the treatment of herniated vertebral discs.

The DNA sequence sequence coding for human stromelysin as described in Figure 4 may be used to design DNA probes for use in

id ntifying the over expression of stromelysin indicative of connective tissue disease states and the invention extends to such DNA probes.

In a twelvth aspect the invention provides a DNA hybridisation probe comprising a sequence of nucleotides selected from the nucleotide sequence from 1 to 1810 as shown in Figure 4 of the accompanying drawings.

In a further aspect the invention provides an antibody having specificity for an antigenic determinator of a mammalian stromelysin. The antibody may be a polyclonal or monoclonal antibody. The antibody may be labelled with a detectable label.

Stromelysin is shown to be able to enhance the activity of collagenase after its activation with, for example, trypsin or APMA and the invention extends to the use of stromelysin for this purpose.

Brief Description of the Drawings

The invention is further described by the following non-limiting examples which refer to the accompanying diagrams Figures 1 to 9 and Tables 1 and 2, short particulars of which are given below.

Figure 1 shows the N. terminal amino acid sequence of rabbit APMA activated stromelysin (a) and the oligo-nucleotide probe based on this sequence. The 50 base oligonucleotide probe was made in two 25 base halves (b and c) and the complementary 26-mer (d) was synthesised in order to facilitate their ligation. The numbering of the amino acids refers to their position in the cDNA predicted rabbit stromelysin sequence (see Fig 2)

Figure 2 Shows the partial nucleotide sequence of rabbit stromelysin and the predicted amino acid sequence

Figure 3 Shows the restriction maps of 3 human stromelysin cDNAs

Figure 4 Shows the restriction map and nucleotide sequence of human stromelysin cDNA and the predicted amino acid sequence

Figure 5 Shows electrophoretic analysis of the products of activation of stromelysin purified from:

<u>panel A</u> - human gingival fibroblast culture medium.

<u>panel B</u> - that secreted by C127 cells transfected with stromelysin-containing vector

<u>panel C</u> - that secreted by COS cells transfected with stromelysin-containing vector

Figure 6 Shows a graph of the time course of activation of C127 cell secreted prostromelysin by various concentrations of trypsin or APMA

Figure 7 Shows electrophoretic analysis of the products of C127 prostromelysin activation with APMA

Figure 8 Shows a graph of the effect of different amounts of purified human fibroblast prostromelysin or prostromelysin secreted by C127 cells on the activation by trypsin of:

A). procollagenase secreted by transfected C127 cells, and

B). procollagenase purified from human gingival fibroblast culture

Figure 9 Shows electrophoretic analysis of procollagenase from human gingival fibroblast culture medium activated by APMA, APMA + stromelysin, trypsin, or trypsin + stromelysin.

Table 1 Amino acid composition of human prostromelysin

Table 2 Amino acid composition of human stromelysin

Detailed Description of the Embodiments

In the following example, prostromelysin is activated by treatment with 4-amino phenylmercuric acetate to yield mature stromelysin. It will be appreciated, however, that by using alternative methods of cleavage of the proenzyme such as enzymic digestion, different forms of active stromelysin may be produced and these are included within the scope of the present invention.

1.1 THE ISOLATION OF A RABBIT cDNA ENCODING STROMELYSIN

4-aminophenylmercuric acetate (APMA) activated (Cawston and Murphy in: Methods in Enzymology, Volume 80 pp711-722 (1981)) rabbit fibroblast stromelysin was purified from the culture media of calvariae (Galloway, W. A. et al Biochem. J. 209, 741-752 (1983)). It was reduced and carboxymethylated and subjected to N-terminal sequencing by automated Edman degradation on an Applied Biosystems gas-phase sequenator. The 50 base oligonucleotide probe was designed according to previously described rules to be capable theoretically of hybridizing to both rabbit stromelysin and collagenase cDNAs (Grantham R. et al Nucleic Acis Res. 9, r43-r47 (1981) and Lathe, R. J. Molec. Biol. 183, 12252-12258 (1983)). It was synthesized as two continuous 25 mers (b and c, Figure 1) by automated solid-phase phosphotriester chemistry followed by purification by HPLC (Patel T. P. et al Nucleic Acids Res. 10, 5605-5619 (1982)). A complementary 26 mer (d) was also made (see Fig. 1).

Rabbit fibroblasts from explants of normal synovium were grown to confluence in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). After washing they were then maintained in serum-free media for 48 h in the presence of

50-200 ng ml^{-1} 12 - O - tetradecanoylphorbol - 13 - acetate (TPA). mRNA was isolated from approximately 5.10^7 cells by the guanidinium isothiocyanate/hot phenol method (Maniatis et al 1982 in. Molecular Cloning: A laboratory Manual; Cold Spring Harbor laboratory, New York), followed by oligo (dT)-cellulose column chromatography (Aviv and Leder, 1972 PNAS USA 69 1408-1412). Standard procedures were used to synthesise cDNA (Gubler and Hoffman, 1983 Gene 25 263-269) and a library of 75,000 plaques was established by using EcoR1 linkers to ligate the cDNA into λ gt10 essentially as described by Huynh et al in DNA Cloning Vol 1 ed. D. M. Glover, IRL Press p49-78.

The oligonucleotide probe was prepared by first subjecting to a kinase reaction the 25 mer labelled (c) fig 1 and then ligating it to the 25-mer labelled (b) in the presence of the complementary 26-mer (d). The 50-base oligonucleotide probe resulting from the ligation was purified from a denaturing polyacrylamide gel (Maxam A. M & Gilbert W. in Methods in Enzymology, Volume 65, pp 499-560 (1980)) and then kinased to a specific activity of approximately 3.10^8 Ce cpm μg^{-1}.

The plaques were transferred to nitrocellulose filters, denatured, and then prehybridised in 5 x SSC, 5 x Denhardt's solution, 50mM NaH_2PO_4, 100 μgml denatured salmon sperm DNA for 6h at $60^{\circ}C$. Hybridisation was then undertaken for 16h at $40^{\circ}C$ in an identical buffer to which had been added 0.1% SDS and the probe DNA at approximately 4ng ml^{-1}. The filters were then washed in 6 X SSC before a final stringent wash (1 X SSC, 0.1% SDS for 15 min at $50^{\circ}C$) followed by autoradiography for 16 h with an intensifying screen. 10 putatively positive plaques were identified of which one gave positive signals after a second screening undertaken as described above. DNA was isolated and the cDNA insert was subcloned into plasmid pSP64 (Melton D. A. et al Nucleic Acids Res. 12, 7035-7056 (1984)). The complete nucleotide sequence of the cDNA was determined by the dideoxy method (Sanger F., Nicklen S. & Coulson A. R. Proc. Natl. Acad. Sci USA 74 5463-5467 (1977)) after subcloning restriction fragments into M13 and transformation into JM101 (Messing J. & Vieira, J. Gene 19 269-276 (1982)).

As shown in fig 2 the cDNA is 532 nucleotides in length and although lacking a poly A tract does have an open reading frame encoding 170 amino acids. Residues 101-127 are identical with the N-terminal amino acid sequence of rabbit stromelysin (Fig 1a) and the amino acid sequence encoded by the cDNA therefore represents the N-terminal third of rabbit stromelysin. This cDNA was therefore used as a probe to identify the human stromelysin cDNA. It was labelled with ^{32}P α dATP and Klenow fragment using the random hexanucleotide method (Feinberg and Vogelstein, 1983, Anal. Biochem, 32 6-13) to a specific activity of 2.10^{7} Ce cpm μg^{-1}.

1.2 ISOLATION OF A HUMAN cDNA ENCODING STROMELYSIN

Human fibroblasts derived from gingival explants (passage 3 to 6) were cultivated in DMEM supplemented with 0.2% lactalbumin hydrolysate and 5% partially purified pig IL-1. After 48h mRNA was isolated from approximately 5×10^{8} cells by the guanidinium isothiocyanate/CsCl method (Maniatis et al 1982 in Molecular Cloning: A laboratory Manual; Cold Spring Harbor Laboratory, New York). The mRNA was used to generate a library of 200,000 recombinant plaques in λgt10 essentially as described above. The plaques were transferred to nitrocellulose, denatured, and then prehybridised in 6 X SSC, 50% formamide, 5 X Denhardt's solution, 100 $\mu g\ ml^{-1}$ denatured salmon sperm DNA for 16 h at 42°C. They were then hybridised for 48h at 42°C in an identical buffer to which had been added 0.1% SDS and the rabbit stromelysin probe cDNA at a final concentration of approximately 17 ng ml^{-1}. The filters were then washed with the highest stringency being 2 X SSC, 0.1% SDS at 42°C for 30 minutes before autoradiography with an intensifying screen for 48h. 21 putatively positive plaques were identified of which 16 gave a clear positive signal after a second screening undertaken as described above. The cDNA insert of representative plaques was isolated and subcloned into plasmid pSP64 (Melton D. A. et al Nucleic Acids Res. 12, 7035-7056 (1984)). Restriction mapping of the largest inserts confirmed that they were related overlapping DNA segments (Fig. 3) and their nucleotide sequence was compiled by the dideoxy method (Sanger F., Nicklen S. &

Coulson A. R. Proc. Natl. Acad. Sci USA 74 5463-5467 (1977)) after subcloning restriction fragments int M13 and transformation into JM101 (Messing J. & Vieira, J. Gen, 19, 269-276 (1982)). This information is shown in Fig 4, where the solid arrows indicate direction of dideoxy sequencing and the broken arrows show directions of sequencing by the Maxam and Gilbert method (Maxam, A.M. and Gilbert, W., Methods Enzymol. (1980) 65 499-560).

1.3 IDENTIFICATION OF RAT cDNA ENCODING STROMELYSIN

We have discovered a high degree of homology between rabbit stromelysin and another amino acid sequence predicted from a rat cDNA, referred to as pTR1 (Matrisian L. M. et al EMBO Journal 4, 1435-1440 (1985)). This cDNA corresponds to a mRNA which is highly enriched in normal fibroblasts after infection with either polyoma virus or rous sarcoma virus or transfection with either the middle t oncogene or the cellular oncogene H-ras. The same mRNA was also reported to be specifically induced after exposure of fibroblasts to EGF (Matrisian L. M. et al EMBO Journal 4, 1435-1440 (1985)). The predicted Mr of the rat protein encoded by pTR1 is approximately 53000, in close agreement with the in vitro translation product of rabbit stromelysin mRNA (Frisch S. M., Chin J. R. & Werb Z. J. Cell Biol. 97, (2, Pt. 5): 430a (Abstr.) (1983)), and data not shown]. We conclude therefore, that the protein encoded by pTR1 is rat stromelysin. The identification of pTR1 as rat stromelysin allows, for the first time, the production of rat stromelysin using recombinant DNA techniques.

2. NUCLEOTIDE SEQUENCE OF HUMAN STROMELYSIN cDNA AND THE PREDICTED AMINO ACID SEQUENCE

The cDNA is 1825 nucleotides in length and appears to be complete at the 3' end because it has a poly A tail preceded by the polyadenylation signal AATAAA (Proudfoot and Brownlee, 1981, Nature, 252 359-362). It contains an open reading frame (nucleotides 51 to 1481) which translates into a polypeptide of 477 amino acids. Evidence that the cDNA encodes stromelysin comes from the finding that 24 of the 27 amino acids encoded by nucleotides 348 to 428 are

identical with and in the sam position as the 27 amino acids identified by N-terminal sequencing the APMA activated rabbit stromelysin (Fig 1a and Fig 4). Furthermore most of the amino acids in the N-terminal third of the molecule are identical with and in the same position as those amino acids predicted from the rabbit partial stromelysin cDNA.

Hydrophobicity plots suggest that human stromelysin is rather water soluble except for a hydrophobic N-terminal sequence of 17 amino acids. This is consistent with these amino acids being the hydrophobic core of a signal sequence which is cleaved during secretion to liberate mature protein. Since it is thought that the metalloproteinases are secreted as proenzymes (Harris E. D. et al Collagen Rel Res 4 493-512 (1984)) we conclude that the amino acids found between the signal sequence and the phenylalanine (F) at the N-termini of the APMA - activated form of rabbit stromelysin (residue 100 in the human stromelysin sequence) are cleaved off during activation. Preprostromelysin is therefore considered to be comprised of amino acids 1-477, prostromelysin is considered to be comprised of amino acids 18-477 and stromelysin is considered to be comprised of amino acids 100-477. The amino acid composition of prostromelysin and stromelysin together with the molecular weight of the core proteins is shown in tables 1 and 2 respectively. Potential glycosylation sites within the sequences exist, and it is envisaged that expression of these proteins in appropriate cells (see below) may give rise to glycosylated products.

3. THE PRODUCTION OF STROMELYSIN

3.1 EXPRESSION IN E. COLI

The expression of stromelysin or prostromelysin with an additional NH_2-terminal methionine residue (met-stromelysin or met-prostromelysin) in E. coli can be achieved by using oligonucleotide linkers to join the mature stromelysin or prostromelysin encoding sequence described in Section 2 above and shown in Figure 4 with a promoter, Shine Dalgarno sequence and an initiating ATG codon. The E. coli trpE pr moter and Shine Dalgarno sequence may be used.

TABLE 1

Amino Acid Composition of Human Prostromelysin

Residue	Number	%	Weight	%
F	32	6.96	4704	9.02
L	38	8.26	4294	8.23
I	20	4.35	2260	4.33
M	6	1.30	786	1.51
V	28	6.09	2772	5.31
S	27	5.87	2349	4.50
P	36	7.83	3492	6.69
T	27	5.87	2727	5.23
A	27	5.87	1917	3.68
Y	17	3.70	2771	5.31
H	13	2.83	1781	3.41
Q	9	1.96	1152	2.21
N	16	3.48	1824	3.50
K	32	6.96	4096	7.85
D	36	7.83	4140	7.94
E	30	6.52	3870	7.42
C	3	0.65	309	0.59
W	8	1.74	1488	2.85
R	23	5.00	3588	6.88
G	32	6.96	1824	3.50
TOTAL	460		52162	

TABLE 2

Amino Acid Composition of Human Stromelysin

Residue	Number	%	Weight	%
F	30	7.94	4410	10.31
L	30	7.94	3390	7.92
I	19	5.03	2147	5.02
M	3	0.79	393	0.92
V	19	5.03	1881	4.40
S	24	6.35	2088	4.88
P	32	8.47	3104	7.25
T	24	6.35	2424	5.67
A	25	6.61	1775	4.15
Y	13	3.44	2119	4.95
H	12	3.17	1644	3.84
Q	6	1.59	768	1.79
N	14	3.70	1596	3.73
K	22	5.82	2816	6.58
D	28	7.41	3220	7.53
E	25	6.61	3225	7.54
C	2	0.53	206	0.48
W	8	2.12	1488	3.48
R	17	4.50	2652	6.20
G	25	6.61	1425	3.33
TOTAL	378		42789	

These sequences are present for example on plasmid pCT54 into which DNA sequences encoding proteins of interest can be inserted in a ClaI site (Emtage et al (1983), Proc. Nat. Acad. Sci. USA, 80, 3671-3675). However, other plasmids also harbouring these or other promoter and Shine Dalgarno sequences but which are amplifiable and from which expression can be tightly regulated may also be used (see, for example, European patent application EP-A2-0121386). The stromelysin or prostromelysin coding sequence without the signal coding sequence but with an additional N-terminal methionine codon, may be inserted into the E. coli expression vector pMG196. This may be achieved by standard recombinant DNA techniques (Maniatis et al (1982), In: Molecular Cloning: A Laboratory Manual, 390-433). Expression of stromelysin or prostromelysin may also be achieved using analogous techniques to those described for expression of TIMP in European patent application No. 86300042.8.

3.2 PURIFICATION OF STROMELYSIN FROM E. COLI CELLS

E. coli cells harbouring a plasmid constructed as described above, when grown under optimal conditions for expression, will produce met-stromelysin or met-prostromelysin at levels of up to 10% of total cellular protein.

The soluble protein fraction from crude E. coli extracts is assayed for stromelysin activity essentially as described by Galaway et al (1983) Biochem. J. 209 741-752. A part or whole of the met-stromelysin expressed in E. coli may be in an insoluble form and therefore not detected in the above assay. In such circumstances, it is solubilised and activated prior to assay and purification. One example of how this can be achieved in relation to methionine-prochymosin production is described in our co-pending International patent application PCT/GB 83/00152 (published as WO 83/04418) and in published British patent application GB 2100737A. Having obtained soluble, active, partially purified stromelysin it is further purifi d using standard protein purification techniques, as described for example by Galaway et al (1983) Biochem. J. 209 741-752.

3.3 EXPRESSION IN YEAST

Using standard recombinant DNA techniques in a similar manner to those used to achieve expression in *E. coli*, plasmid vectors suitable for expression of stromelysin in yeast are constructed. The constructions are based, for example, on the vectors described in co-pending published European patent application EP-A2-0073635. They contain the stromelysin or prostromelysin encoding sequence flanked by the yeast phosphoglycerate kinase (PGK) promoter and the PGK gene 3' untranslated end. The orientation of the stromelysin or prostromelysin cDNA with respect to the PGK promoter is such that it ensures expression of mature stromelysin or prostromelysin with an additional NH_2-terminal methionine residue as described for expression in *E. coli*. Alternatively, pre-stromelysin or prostromelysin may be expressed by joining the stromelysin or prostromelysin encoding sequence to the vector such that the stromelysin signal sequence is left in place. Expression of a fusion protein between a yeast signal sequence (for example, the yeast α-Factor signal sequence (Kurjan and Herskowitz, (1982), Cell, 30, 933-943), and mature stromelysin or prostromelysin can be achieved through the use of appropriate linkers. These plasmid DNAs are introduced into yeast cells, for example, by the method of Beggs (Nature, (1978), 275, 104-109).

3.4 PURIFICATION OF STROMELYSIN FROM YEAST CELLS

Yeast cells containing these plasmids, when grown under optimal conditions for stromelysin expression, will produce up to 5% of total cellular protein as stromelysin or prostromelysin. Depending on the alternatives described above, stromelysin or prostromelysin may or may not be secreted from the yeast cells. Expressed stromelysin is quantified, assayed and purified essentially as described above if produced intercellularly. If secreted it is purified from cell supernatants by standard protein purification techniques (Galaway *et al* (1983) Biochem. J. 209 741-752).

3.5 EXPRESSION OF STROMELYSIN IN CULTURED ANIMAL CELLS

The preprostromelysin encoding cDNA was inserted into the different kinds of expression vector designed specifically for: 1. transient expression in COS cells (Gluzman, Y. (1981) Cell 23 175-182) and 2. Stable expression in C127 cells (Lowy, D.R. et al J. Virol. (1978) 26 291-298). The necessary DNA manipulations employed standard techniques (Maniatis et al In. Molecular Cloning, A Laboratory Manual, 1982, Cold Spring Harbor Laboratory, New York). The cDNA was tailored for insertion into the vectors by first converting the Ava I site encoded by nucleotides 1591-1596 (Figure 4) into an EcoR1 site. This was achieved by cleaving the cDNA with Ava I, filling in with T4 polymerase followed by addition of an EcoR1 linker. On cleavage with EcoR1 a 1600 bp EcoR1 fragment encoding preprostromelysin extending from the 5' EcoR1 site encoded by nucleotides 1-6 in Figure 4 to the newly created EcoR1 site is generated. This fragment was purified from a 1% low gelling temperature agarose gel and then ligated between the SV40 late promoter and the SV40 early polyadenylation regulatory elements in a "poison minus" derivative of a pBR322 COS cell vector (Lusky, M and Botchan, M.R., Nature, 1981, 293 79-81). Vector DNA containing the preprostromelysin sequence in the appropriate orientation with respect to the SV40 late promoter was purified from cesium chloride gradients and transfected into COS cells using DEAE dextran (Lopata, M.A. et al Nucleic Acids Res., 1984, 12 5707-5717). Serum-free supernatants were harvested 72 hours after transfection.

For expression in C127 cells the preprostromelysin sequence with the early SV40 polyadenylation regulatory elements was removed on a Pst1 to BamH1 fragment from the COS cell vector and put under the control of the mouse metallothionine I promoter in a bovine papilloma virus (BPV) based vector. This arrangement was essentially as described for the expression of tissue inhibitor of metalloproteinase (TIMP), Docherty et al Nature 1985, 318 66-69 and UK Patent Application GB 2169295A. The BPV vector encoding preprostromelysin was introduced into C127 cells by the calcium ph sphate coprecipitati n method (Wigler, M. et al 1978, Cell 14 725-731) and $CdCl_2$ (20μM) - and $ZnCl_2$ (20μM) - resistant

foci were selected. Str melysin producing cell lines were identified after 21 days and used to generate serum-free culture medium.

4. STROMELYSIN ACTIVATION, ASSAY AND ELECTROPHORETIC ANALYSIS

The stromelysin produced as described above was activated with either trypsin or APMA. Trypsin was incubated with the stromelysin at ratios such as 1:100 to 1:1 respectively followed by the addition of a ten-fold excess of soya-bean trypsin inhibitor. Concentrations of trypsin such as 0.1 to 10μg ml^{-1} were employed and the temperatures and time of incubation were as described below. Treatment with 4-aminophenylmercuric acetate (APMA) was at 1 to 2 mM for various times as indicated below, at 37°C. Stromelysin activity was assayed using ^{14}C-acetylated casein (Galloway, W.A., et al Biochem. J. 1983, 209 741-752) at 35°C for 1 or 4 h. 1 unit of stromelysin degrades 1μg of casein min^{-1} at 37°C.

Stromelysin before and after activation as described above and in more detail below was treated with 20mM EDTA, reduced with 500mM 2-mercaptoethanol and run on 10% polyacrylamide mini gels in the presence of SDS. Gels were electro-transferred to nitrocellulose and the enzyme bands visualised using an antisera against rabbit stromelysin which was able to detect human stromelysin (Murphy, G. et al Collagen Rel. Res. 1986 6 351-364) and a peroxidase-labelled second antibody (Hembry, R.M., et al J. Cell Sci. 1985 73 105-119).

As shown in Figure 5, prostromelysin preparations A, purified from human gingival fibroblast culture medium, B, secreted by C127 cells transfected with the stromelysin-containing vector and C, secreted by COS cells transfected with the stromelysin-containing vector, were treated as follows: 1. no treatment; 2. incubation with 1mM APMA for 2h at 37°C; 3. incubation with 10/μg/ml trypsin for 30 min at 37°C; 4. as 3. with addition of soya bean trypsin inhibitor after 30 min and further incubation for 2h at 37°C; 5. incubation with 100μg/ml trypsin for 10 min at 4°C; 6. incubation with 10μg/ml trypsin for 5 min at 4°C;

7. as 6. with addition of soya bean trypsin inhibitor after 5 min and furth r incubation for 2h at 37^{o}C. The samples were electrophoresed on a 10% polyacrylamide gel in SDS and reducing conditions, electro-blotted onto nitrocellulose and visualised using a sheep anti rabbit stromelysin antibody, and a peroxidase labelled rabbit anti sheep lgG.

As shown in Figure 6 prostromelysin secreted by C127 cells transfected with the stromelysin-containing vector was incubated with A, (●) 0.1µg/ml trypsin, (Δ) 1µg/ml trypsin, B, (□) 10µg/ml trypsin, C, (∇) 100µg/ml trypsin at 37^{o}C (closed symbols) or 4^{o}C (open symbols) in a volume of 20µl, or D, (♦) with 2mM APMA at 37^{o}C. Activity elicited was assayed by degradation of ^{14}C-casein in a 1h assay at 37^{o}C, after addition of soya bean trypsin inhibitor. Stromelysin incubated without trypsin or APMA had no activity.

Prostromelysin was activated with APMA (as described in Figure 6) for varying lengths of time. The samples were then treated with 20mM EDTA and electrophoresed under reducing conditions on a 10% polyacrylamide gel containing SDS and electroblotted and visualised as described in Fig. 5 and Methods. Figure 7 shows the SDS analysis where Track 1. 0 min; Track 2. 30 min; Track 3. 1h; Track 4. 2h; Track 5. 4h; Track 6. 15h.

The pro-forms of either purified natural stromelysin from human gingival fibroblasts, or the recombinant enzyme secreted by COS or C127 cells had an identical Mr of 57000, with a minor 60000 component (probably due to glycosylation (Nagase, H. et al (1983) Biochem. J. 214 281-288); Figure 5, lanes A1, B1, C1). These forms were completely inactive against casein or other substrates (Galloway, W.A. et al (1983) Biochem. J. 209 741-752) but could be activated by trypsin, (e.g. C127 stromelysin, Fig. 6A-C) or less efficiently by APMA, to degrade these substrates (Figure 6D). Trypsin activation was optimal over a wide range of concentrations; activation could be effected at 4^{o}C, with marginally slower kinetics (Figure 6A-C). Only very high levels f trypsin inactivated stromelysin. Activation by APMA was slower, achieving 58% (COS) to 90% (C127 and

natural) of th trypsin value. Although maximum APMA activation was attained in 4-8 h at 37°C (Figure 6), it was found that activation was dependent upon stromelysin concentration (73% of optimal at a ten fold dilution and 33% at a one hundred fold dilution of a 50 unit/ml preparation). Hence, very low concentrations of enzyme, such as the COS cell culture media were probably not maximally activated even after 15 h incubation.

Analysis of the effect of these treatments on the prostromelysin by gel electrophoresis and immuno blotting showed that a reduction in Mr occurred to yield two major species of Mr. 50000 and 48000 slowly in the case of APMA (e.g. C127 stromelysin, Figure 7) and very rapidly in the case of trypsin (Figure 5 A3; B3,5; C3,5). At longer incubation times with APMA traces of an Mr. 28000 form of stromelysin were generated (Figure 7). Plasmin could also be used to generate bands of the same Mr as those produced by trypsin (data not shown). It was noted that optimal activation (as documented in Figure 6) did not require complete conversion of the upper doublet to the lower doublet (Figure 5; lanes A6, A7; Figure 3, lane 4). Using collagenase, Stricklin <u>et al</u> (Biochemistry <u>22</u> 63-68 (1983)) have reported similar observations with activity detectable prior to an Mr loss. It has been proposed that conformational changes in the intact molecule occur, leading to subsequent self-cleavage. The activity of stromelysin elicited was 360 units/mg.

5. <u>COLLAGENASE ACTIVATION ELECTROPHORETIC ANALYSIS AND ASSAY</u>

The collagenase was activated as described for stromelysin or as detailed below. Electrophoretic analysis was undertaken as described for stromelysin except that enzyme bands were visualised using anti-human collagenase antisera followed by a peroxidase-labelled second antibody (Hembry, R.M. <u>et al</u> J. Cell Sci. 1985, <u>73</u> 105-119). The collagenase was assayed by the ^{14}C-acetylated collagen diffuse fibril assay at 35°C for 4 hours (Whitham, S.E. <u>et al</u> Biochem. J. 1986, <u>240</u> 913-916). 1 unit of collagenase degrades 1μg of type I collagen min^{-1} at 35°C.

Figure 8 shows the results when procollagenase preparations, A, secreted by C127 cells transfected with the collagenase containing vector and B, purified from human gingival fibroblast culture m dium were incubated with 10µg/ml trypsin at 37°C for 30 min in the presence of varying amounts of (●) purified human fibroblast prostromelysin or (■) prostromelysin secreted by C127 cells transfected with the stromelysin-containing vector, followed by the addition of excess soya bean trypsin inhibitor. Open symbols denote the effect of the corresponding activated stromelysin in the absence of trypsin. Activity was assayed using ^{14}C-type I collagen at 35°C for 4h. Results are expressed as the fold increase elicited by stromelysin relative to the activity with trypsin alone.

Figure 9 shows electrophoretic analysis of procollagenase purified from human gingival fibroblast culture medium treated as follows: 1. no treatment; 2. incubation with 1mM APMA at 37°C for 2h; 3. as 2, in the presence of purified stromelysin; 4. incubation with 10µg/ml^{-1} trypsin at 37°C for 30 min, followed by addition of soya bean trypsin inhibitor; 5. as 4. in the presence of stromelysin.

Purified natural procollagenase has an Mr of 55000 with a minor component of Mr 59000 upon analysis by SDS polyacrylamide gel electrophoresis and blotting with a specific antiserum to collagenase (Figure 9; lane 1). The procollagenase could be activated by either APMA or trypsin treatment behaving precisely as described by Stricklin <u>et al</u> (Biochemistry <u>22</u> 63-68 (1983)) with a fall in Mr of 10,000 (Figure 9, lanes 2 and 4). The activity elicited was very low, in the region of 320 units/mg. A similar observation was made by Vater <u>et al</u> (J. Biol. Chem. <u>258</u> 9374-9382 (1983)) for rabbit procollagenase. However, the inclusion of either purified or recombinant human prostromelysin in the activation mixture enhanced the collagenase activity by up to 8 fold (Figure 8B). Similar effects of stromelysin on the molecular weight and activity of recombinant C127 cell derived human collagenase were also obtained. With the recombinant collagenase the stromelysin brought about a 12 fold enhancement in activity (Figure 8A).

Addition of previously activated stromelysin to the collagenase after trypsin treatment had less effect on the final collagenase activity detectable (data not shown). Active stromelysin alone elicited similar collagenase activities to those with trypsin alone. The ratio of stromelysin:collagenase for efficient activation was in the order of an excess of 2moles or more per mole. It is possible that higher specific activity stromelysin would be a more efficient activator.

Analysis of the changes occurring during these treatments showed that the procollagenase, underwent stepwise reductions in Mr APMA and trypsin generated two bands of about 50000 and 45000 (Figure 9, lanes 2 and 4) which were converted to bands of 48000 and 43000 in the presence of stromelysin (Figure 9; lanes 3 and 5). The same band patterns were generated by both natural and recombinant stromelysin. Activated stromelysin alone appeared to produce very small changes in procollagenase of Mr about 500, with limited further conversion to 48000 and 43000.

CLAIMS

1. A process for the production of a mammalian stromelysin comprising culturing host cells transformed with a DNA sequence coding for the stromelysin.

2. A process for the production of a mammalian stromelysin comprising producing a precursor of the stromelysin by culturing host cells transformed with a DNA sequence coding for the stromelysin precursor and cleaving the precursor to produce the stromelysin.

3. An essentially pure mammalian stromelysin, prostromelysin or preprostromelysin characterised in that the mammalian stromelysin, prostromelysin or preprostromelysin is human stromelysin, prostromelysin or preprostromelysin.

4 A mammalian stromelysin or prostromelysin produced by a process according to Claim 1.

5. A fusion protein comprising a heterologous protein and a mammalian stromelysin or prostromelysin.

6. A DNA sequence coding for the amino acid sequence of a mammalian stromelysin or prostromelysin or a precursor thereof provided that when the DNA sequence codes for rat stromelysin the DNA sequence is not the pTR1 cDNA sequence.

7. An expression vector including a DNA sequence coding for a mammalian stromelysin, prostromelysin or precursor thereof.

8. Host cells transformed with a vector according to Claim 7.

9. A pharmaceutical composition comprising a mammalian stromelysin and a pharmaceutically acceptable excipient.

10. A method of therapy comprising treating a patient with an effective amount of a mammalian stromelysin.

Fig. 1

a. F S T F P G T P K W T K T H L T Y R I V N Y T P D L P
101 ... 127

b. 5'-TGGAACCAGACCCACCTGACCTACAGGATTGTGAACTACACCCCCGACCT-3'
c.

d. GTGGACTGGATGTCCTAACACTTGAT

Fig. 2

```
                         M  K  Y  L  F  T  L  L  L  L  C  V  A  L  C  S  A  Y  F  L  D  G  A  S  R  D  A  D  T  T  N  M  D
CGAGCTGAGTAAAGCCAATGGAAATGAAAACTCTTCCAACCCTGCTACTGCTGTGCGTGGCGCTTTGCTCAGCCTATCCACTGGATGGAGCCTCAAGGGATGCAGACACCACCAACATGG
        10        20        30        40        50        60        70        80        90       100       110       120

  L  L  Q  Q  Y  L  E  N  Y  Y  N  L  E  K  D  V  K  Q  F  V  K  R  K  D  S  S  P  V  V  K  K  I  Q  E  M  Q  K  F  L  G
ACCTTCTTCAGCAATATCTGGAAAACTACTACAACCTTGAAAAAGATGTGAAACAGTTTGTTAAAAGAAAGGACAGTAGTCCTGTTGTTAAAAAAATCCAAGAAATGCAGAAGTTCCTTG
       130       140       150       160       170       180       190       200       210       220       230       240

  L  E  V  T  G  K  L  D  S  N  T  L  E  V  I  R  K  P  R  C  G  V  P  D  V  G  H  F  S  T  F  P  G  T  P  K  W  T  K  T
GCTTGGAGGTGACAGGCAAGCTGGACTCCAACACCCTGGAGGTGATACGCAAGCCCAGGTGTGGCGTTCCTGATGTTGGTCACTTCAGTAC-TTCCCTGGCACCCCAAAGTGGACAAAAA
       250       260       270       280       290       300       310       320       330       340       350       360

  H  L  T  Y  R  I  V  N  Y  T  P  D  L  P  R  D  A  V  D  A  A  I  E  K  A  L  K  V  W  E  E  V  T  P  L  T  F  S  R  K
CTCACCTAACTTACAGGATTGTGAATTACACACCGGATCTGCCAAGAGATGCTGTTGATGCTGCCATTGAGAAAGCTCTGAAAGTCTGGGAGGAGGTGACTCCACTCACATTCTCCAGGA
       370       380       390       400       410       420       430       440       450       460       470       480

  Y  E  G  E  A  D  I  M  I  S  F  G  V  R  G  N  S
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       490       500       510       520       530
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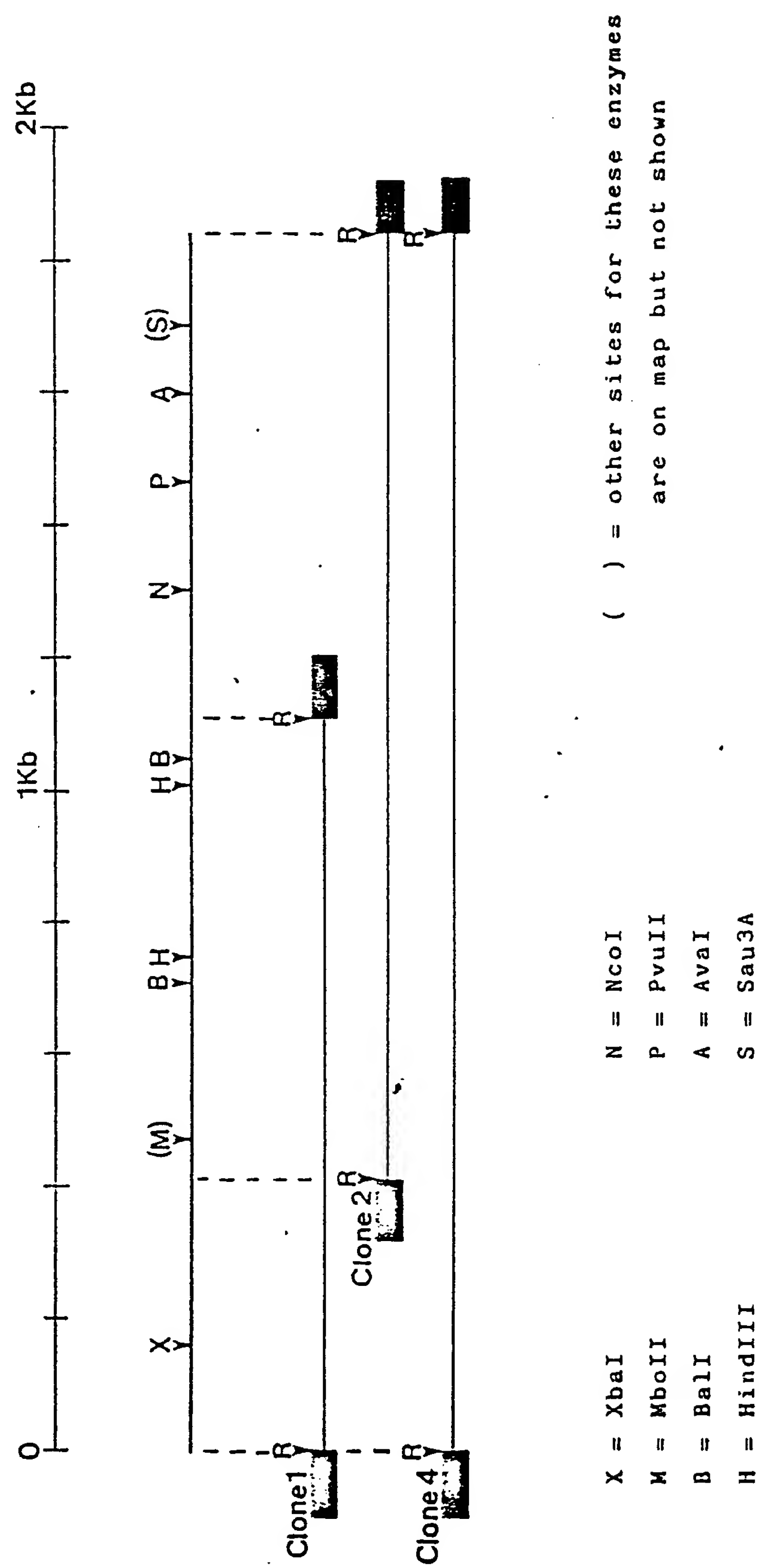
Fig. 3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2Kb
X
(M)
B H
H B
N
P
A
(S)
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Clone1
Clone 2
Clone 4
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M = MboII
B = BalI
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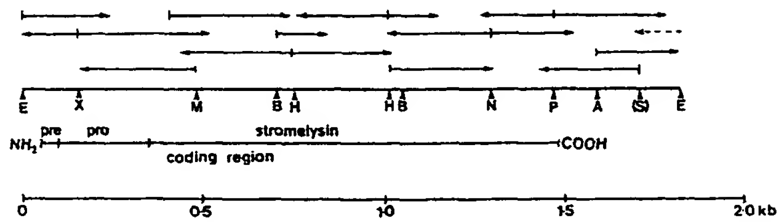
S = Sau3A
( ) = other sites for these enzymes
are on map but not shown

3/7

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                                                        1
101   CTATCCATTGGATGGAGCTGCAAGGGGTGAGGACACCAGCATGAACCTTGTTCAGAAATATCTACAAAACTACTACGACCTCAAAAAAGATGTGAAACAG   200
       Y  P  L  D  G  A  A  R  G  E  D  T  S  M  N  L  V  Q  K  Y  L  E  N  Y  Y  D  L  K  K  D  V  K  Q
      18
201   TTTGTTAGGAGAAAGGACAGTGGTCCTGTTGTTAAAAAAATCCGAGAAATGCAGAAGTTCCTTGGATTGGAGGTGACGGGGAAGCTGGACTCCGACACTC   300
      F  V  R  R  K  D  S  G  P  V  V  K  K  I  R  E  M  Q  K  F  L  G  L  E  V  T  G  K  L  D  S  D  T  L
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                                                    100
401   GATTGTGAATTATACACCAGATTTGCCAAAAGATGCTGTTGATTCTGCTGTTGAGAAAGCTCTGAAAGTCTGGGAAGAGGTGACTCCACTCACATTCTCC   500
       I  V  N  Y  T  P  D  L  P  K  D  A  V  D  S  A  V  E  K  A  L  K  V  W  E  E  V  T  P  L  T  F  S
501   AGGCTGTATGAAGGAGAGGCTGATATAATGATCTCTTTTGCAGTTAGAGAACATGGAGACTTTTACCCTTTTGATGGACCTGGAAATGTTTTGGCCCATG   600
      R  L  Y  E  G  E  A  D  I  M  I  S  F  A  V  R  E  H  G  D  F  Y  P  F  D  G  P  G  N  V  L  A  H  A
601   CCTATGCCCCTGGGCCAGGGATTAATGGAGATGCCCACTTTGATGATGATGAACAATGGACAAAGGATACAACAGGGACCAATTTATTTCTCGTTGCTGC   700
        Y  A  P  G  P  G  I  N  G  D  A  H  F  D  D  D  E  Q  W  T  K  D  T  T  G  T  N  L  F  L  V  A  A
701   TCATGAAATTGGCCACTCCCTGGGTCTCTTTCACTCAGCCAACACTGAAGCTTTGATGTACCCACTCTATCACTCACTCACAGACCTGACTCGGTTCCGC   800
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        V  R  K  I  D  A  A  I  S  D  K  E  K  N  K  T  Y  F  F  V  E  D  K  Y  W  R  F  D  E  K  R  N  S
1301  CATGGAGCCAGGCTTTCCCAAGCAAATAGCTGAAGACTTTCCAGGGATTGACTCAAAGATTGATGCTGTTTTTGAAGAATTTGGGTTCTTTTATTTCTTT   1400
        M  E  P  G  F  P  K  Q  I  A  E  D  F  P  G  I  D  S  K  I  D  A  V  F  E  E  F  G  F  F  Y  F  F
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      T  G  S  S  Q  L  E  F  D  P  N  A  K  K  V  T  H  T  L  K  S  N  S  W  L  N  C
                                                                                  477
1501  GCACAATATGGGCACTTTAAATGAAGCTAATAATTCTTCACCTAAGTCTCTGTGAATTGAAATGTTCGTTTTCTCCTGCCTGTGCTGTGACTCGAGTCAC   1600
1601  ACTCAAGGGAACTTGAGCGTGAATCTGTATCTTGCCGGTCATTTTTATGTTATTACAGGGCATTCAAATGGCTGCTGCTTAGCTTGCACCTTGTCACAT   1700
1701  AGAGTGATCTTTCCCAAGAGAAGGGGAAGCACTCGTGTGCAACAGACAAGTGACTGTATCTGTGTAGACTATTTGCTTATTTAATAAAGACGATTTGTCA   1800
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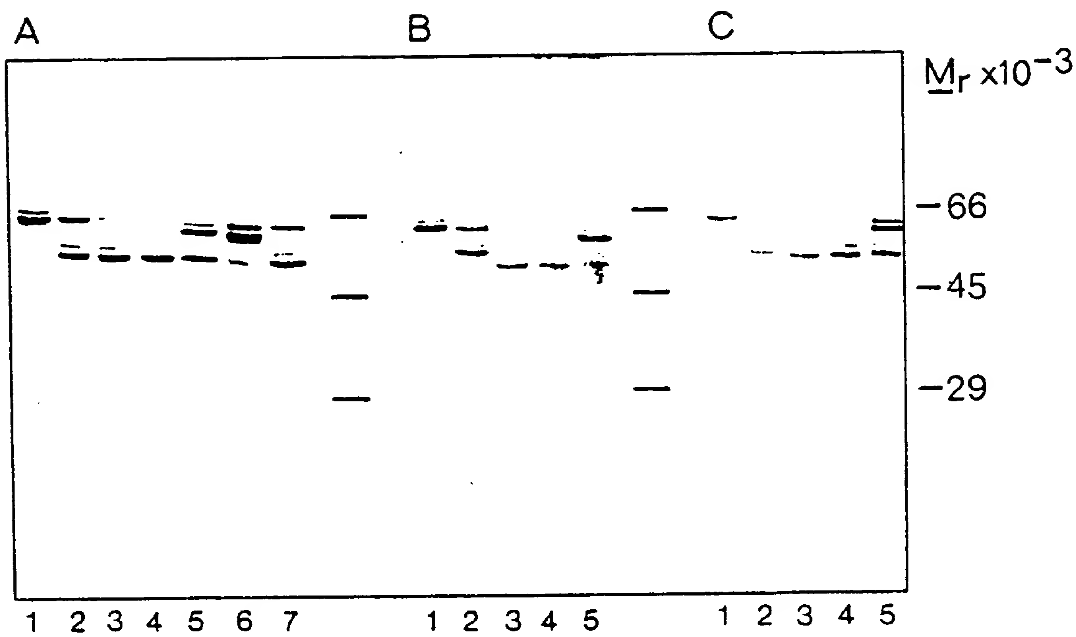
Fig. 4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B
C
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–45
–29
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1 2 3 4 5
1 2 3 4 5

Fig. 5

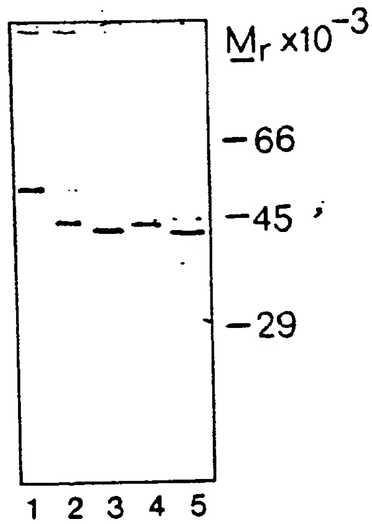
$\underline{M}_r$ x10^{-3}
–66
–45
–29
1 2 3 4 5

Fig. 9

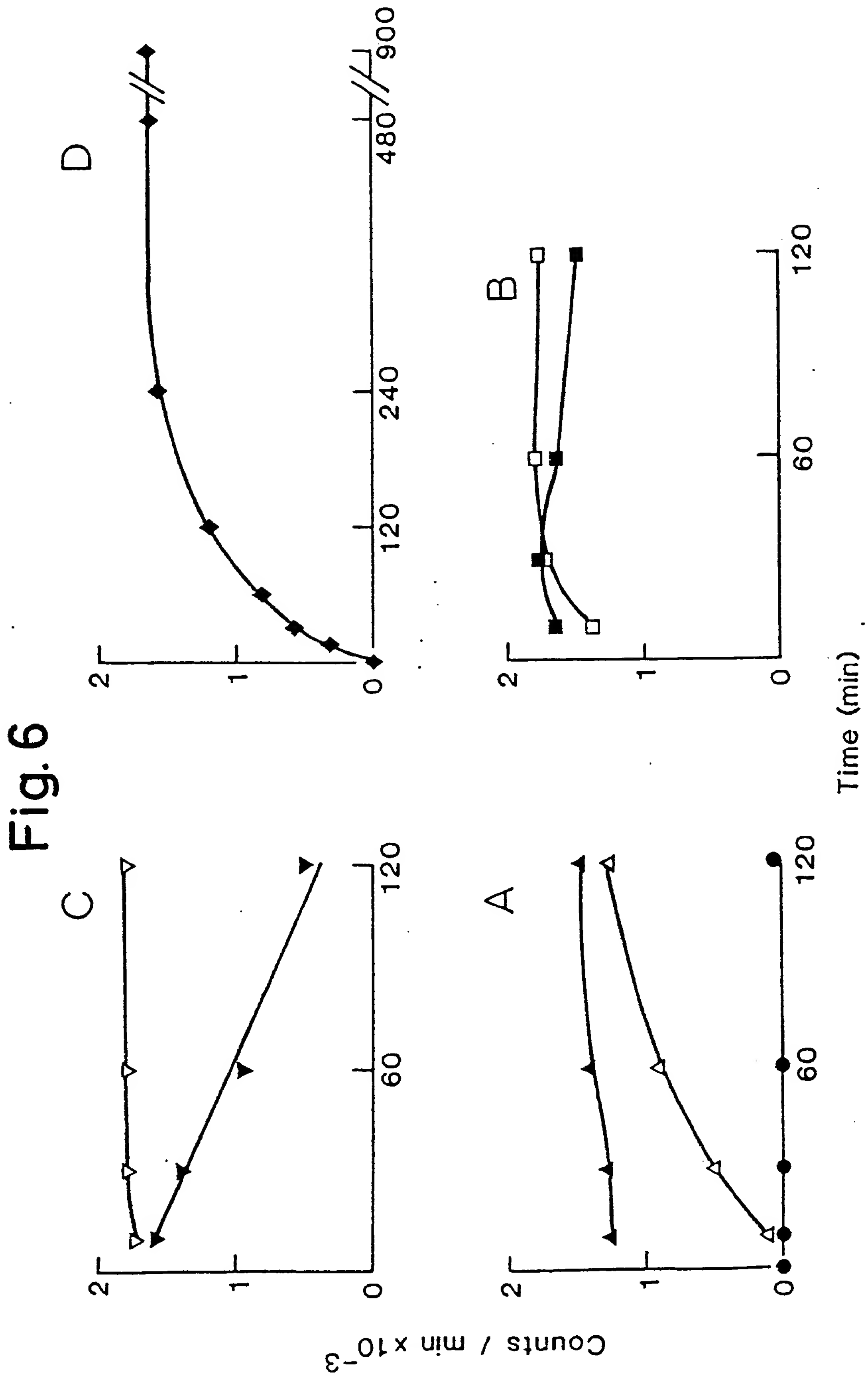

Fig.6

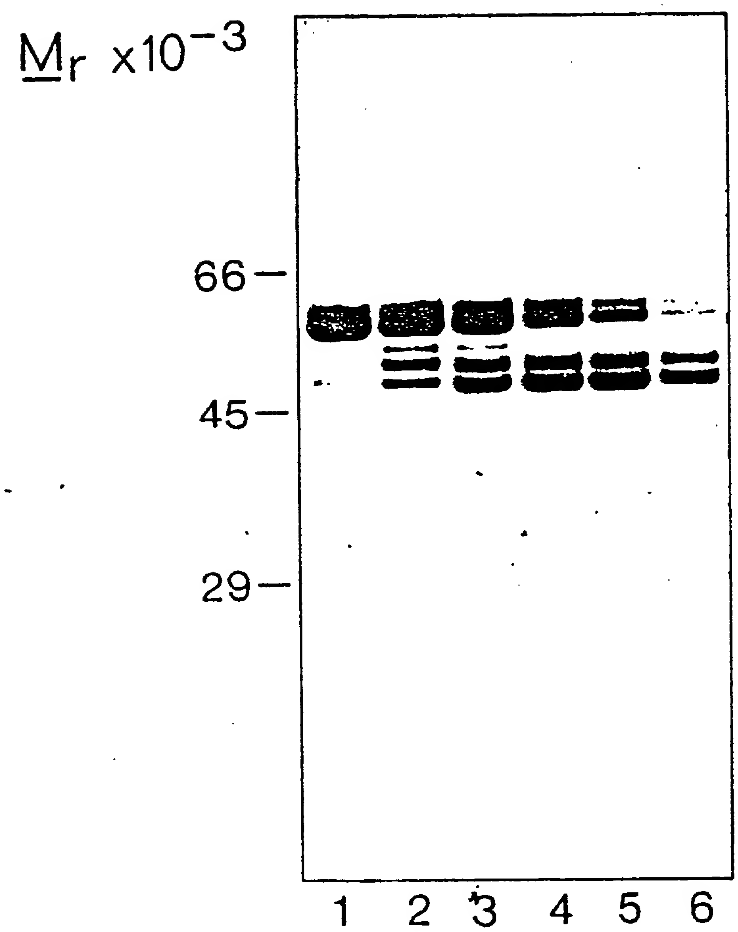

Fig. 7

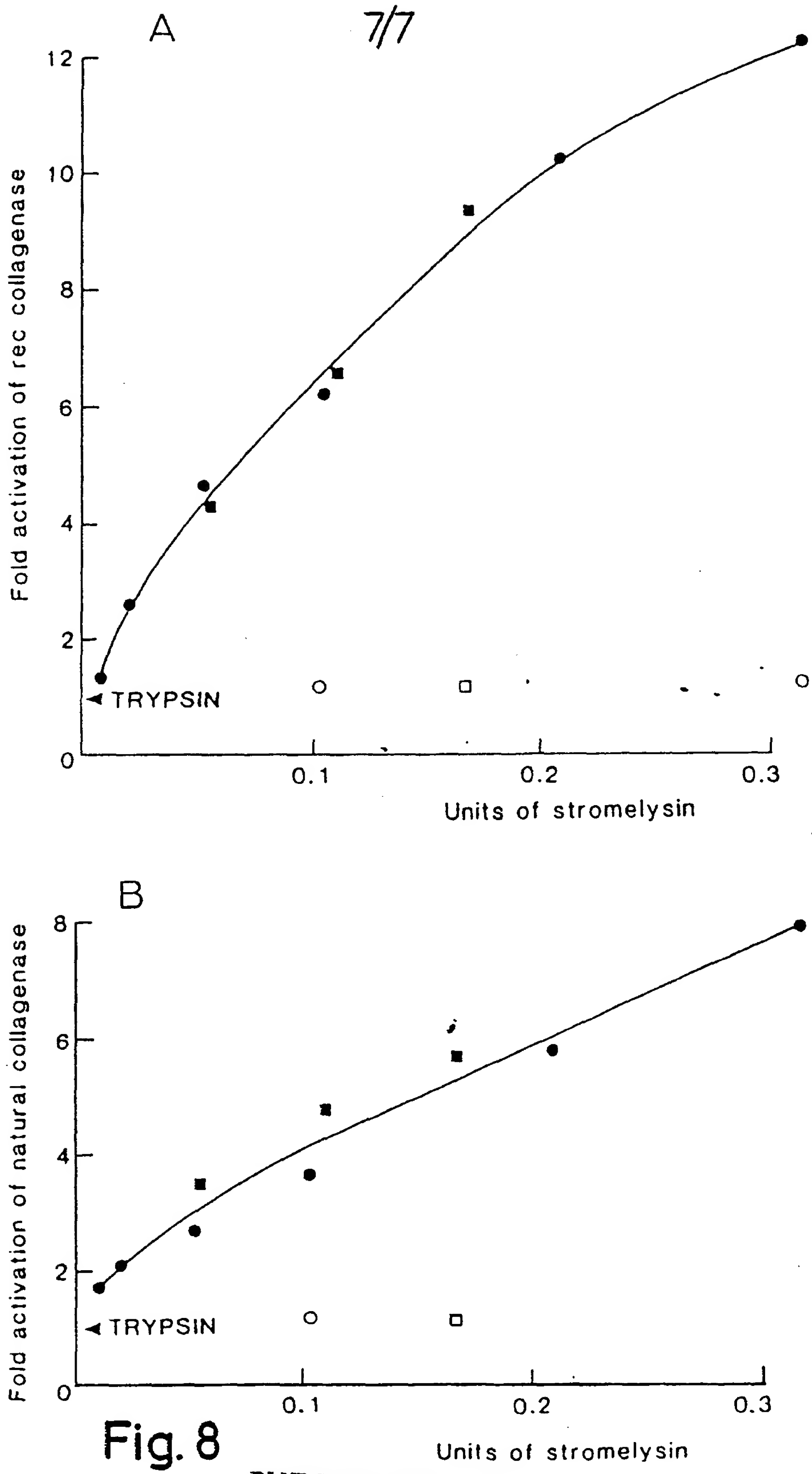
A
12
10
8
6
4
2
0
Fold activation of rec collagenase
TRYPSIN
0.1
0.2
0.3
Units of stromelysin
B
8
6
4
2
0
Fold activation of natural collagenase
TRYPSIN
0.1
0.2
0.3
Units of stromelysin

Fig. 8

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 87/00420

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC^4: C 12 N 15/00; C 12 N 9/64; A 61 K 37/54; C 12 N-1/00

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC^4	C 12 N A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [*]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X Y	The Journal of Biological Chemistry, volume 260, no. 22, 5 October 1985, The American Society of Biological Chemists, Inc., (US), J.R. Chin et al.: "Stromelysin, a connective tissue-degrading metallo-endopeptidase secreted by stimulated rabbit synovial fibroblasts in parallel with collagenase", pages 12367-12376 see the whole document --	3,4 1,2,6-8
X Y	Biochem. J., volume 209, 1983, The Biochemical Society, (GB), W.A. Galloway et al.: "Purification and characterization of a rabbit bone metalloproteinase that degrades proteoglycan and other connective-tissue components", pages 741-752 see the whole document cited in the application -- ./.	3,4 1,2,6-8

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
6th October 1987	19 NOV 1987
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	M. VAN MOL

III. DOCUMENTS CONSIDERED T BE RELEVANT (C NTINUED FR M THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	The EMBO Journal, volume 4, no. 6, 1985, IRL Press Limited, (Oxford, GB), L.M. Matrisian et al.: "Epidermal growth factor and oncogenes induce transcription of the same cellular mRNA in rat fibroblasts", pages 1435-1440 see the whole document cited in the application	 1,2,6-8
X Y	J. Cell Biol., volume 97, no. 2, part 5, 1983, S.M. Frisch et al.: "Molecular cloning of a cDNA encoding a secreted connective tissue-degrading metallo-proteinase induced by changes in cytoskeletal structure", page 460a, abstract 1740 see the whole abstract cited in the application	 6 1,2,7,8
P,X	Biochem. J., volume 240, 1986, (GB), S.E. Whitham et al.: "Comparison of human stromelysin and collagenase by cloning and sequence analysis", pages 913-916 see the whole document	 6
Y	EP, A, 0157604 (SANKYO CO., LTD) 9 October 1985 see the whole document	 1,2,6-8
P,X	Journal of Cell Biology, volume 103, no. 5, part 2, 1986, (US), E.J. Clark et al.: "Synthesis and secretion of collagenase and stromelysin in SV40 transformed human ataxia-telangiectasia fibroblasts", page 257a, abstract 961 see the whole abstract	 6
A	Chemical Abstracts, volume 105, no. 17, 27 October 1986, (Columbus, Ohio, US), S.M. Frisch: "Molecular biology of the stromelysin and collagenase genes", see page 316, abstract 148779t, & Diss. Abstr. Int. B 1986, 46(5), 1544	
A	Chemical Abstracts, volume 105, no. 3, 21 July 1986, (Columbus, Ohio, US), G.I. Goldberg et al.: "Human fibro-blast collagenase. Complete primary structure and homology to an oncogene transformation-induced rat protein",./.	

International Application No. PCT/GB 87/00420

FURTHER INF RMATION CONTINUED FR M THE SECOND SHEET

	see page 209, abstract 19690h, & J. Biol. Chem. 1986, 261(14), 6600-5	
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P,A	GB, A, 2182665 (MONSANTO CO.) 20 May 1987	9,10
	--	
A	EP, A, 0115974 (INSTITUT PASTEUR) 15 August 1984 see the whole document	
	--	
A	EP, A, 0159497 (K.K. YAKULT HONSHA) 30 October 1985 see the whole document	9,10

V.[X] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1.[X] Claim numbers ....10... because they relate to subject matter not required to be searched by this Authority, namely:

See PCT rule 39.1 (IV) Methods for treatment of the human or animal body by surgery or therapy, as well as diagnostic methods.

2.[ ] Claim numbers ...., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3.[ ] Claim numbers.........., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI.[ ] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1.[ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2.[ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3.[ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4.[ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

[ ] The additional search fees were accompanied by applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON

INTERNATIONAL APPLICATION NO. PCT/GB 87/00420 (SA 17576)

This Annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 22/10/87

The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A- 0157604	09/10/85	JP-A-	60207583	19/10/85
GB-A- 2182665	20/05/87	None		
EP-A- 0115974	15/08/84	WO-A-	8402653	19/07/84
		FR-A-	2538702	06/07/84
		AU-A-	2410584	02/08/84
		JP-T-	60500413	28/03/85
		FR-A,B	2538701	06/07/84
EP-A- 0159497	30/10/85	JP-A-	60188066	25/09/85
		US-A-	4624924	25/11/86

For more details about this annex :
see Official Journal of the European Patent Office, No. 12/82